Postersession

Crosslinking of cartilage matrix protein to aggrecan increases with maturation

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Cartilage matrix protein (CMP) is a trimeric protein occurring in some types of cartilage extracellular matrix (Paulsson and Heinegård 1982). It has recently been purified under native conditions which allowed the proposal of a structural model (Hauser and Paulsson 1994). Early data indicated an interaction of CMP with aggrecan. We now describe the isolation of a complex between CMP and aggrecan from tissue extracts. Proteoglycans prepared from cartilage using conditions minimizing contamination by unrelated proteins consistently contained CMP, apparently associated with the core protein and only partially released upon reduction, even under denaturing conditions. It appears that CMP binds by a nonreducible covalent interaction with one of its subunits to the protein core. Electron microscopy revealed interaction sites for CMP with core protein in the extended domain E2.

Materials and methods

Fresh bovine tracheal cartilage from a fetus at four months of gestation and steers at the age of 3–6 months, 18 months and 6 years were obtained from the local slaughterhouse. Samples were prewashed with TBS and homogenized in 10 volumes of 4 M guanidine HCl, 0.01 M Tris/HCl, pH 7.4, containing 2 mM PMSF, 2 mM NEM, and 10 mM EDTA and extracted overnight at 4 °C.

Aggrecan was purified by CsCl density gradient centrifugation in the presence of 4 M guanidine HCl. All fractions were analyzed for CMP by SDS-PAGE and immunoblotting. D1 fractions from the 3–6 months and 6 years old cartilage were applied to a Sepharose CL-4B molecular sieve, eluted with 4 M guanidine HCl, 0.05 M Tris/HCl, 10 mM EDTA, pH 7.4. Void volume fractions were pooled and digested with chondroitinase ABC and keratanase. Digested and untreated samples were analyzed by SDS-PAGE and immunoblotting with antibodies against CMP and aggrecan G1-domain. The digested samples were further purified by gel chromatograpy on Superose 6, eluted with the same solvent as above. Void volume fractions, enriched in aggrecan-CMP complexes, were pooled and subjected to glycerol spraying/rotary shadowing electron microscopy.

Results and Discussion

CMP-aggrecan complexes from bovine tracheal cartilage of different ages (fetuses to 6 year old animals) were isolated by extraction and isopycnic CsCl gradient centrifugation in the presence of 4 M guanidine HCl. In fetal and immature cartilage most of the CMP was found in the top fractions (D5), while with increasing age the distribution shifted towards the high buoyant density aggrecan-rich D1 fractions. The D1-fractions were further purified by gel filtration, where CMP-aggrecan complexes were enriched in the void volume fractions.

Immunoblotting of aggrecan from old cartilage showed that CMP remained bound to the core protein even after digestion with chondroitinase ABC and keratanase. This indicates that CMP is bound directly to the core rather than to the glycosaminoglycan side chains. Corresponding samples from immature cartilage did virtually not stain for CMP, even though the staining pattern for the G1-domain was similar to that of the old cartilage, showing further evidence for an increase of the tight association between CMP and protein core with maturation and aging. SDS-PAGE under reducing conditions of core protein from old cartilage, followed by immunolocalization of CMP, showed positive staining both in the position of core protein and free CMP subunits. Thus, it appears that not all the subunits of CMP are directly bound to the core protein.

Electron microscopy showed that the proportion of core protein carrying bound CMP increased with aging. The distribution of binding sites on the core varied with age. Additional binding sites for CMP were found on the protein cores from old cartilage.

Binding sites for CMP were more abundant and the extent of decoration with CMP increased with aging.

It appears that several pools of CMP are found in the cartilage matrix. These may contribute different properties and functions. One pool represents tightly bound CMP that can not be released by detergents, chaotropic agents nor by reduction. Upon maturation and aging of the tissue a marked increase in total CMP-content is accompanied by a characteristic shift from unbound to tightly bound CMP.

References

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Distribution of CMP and COMP in human cartilage

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Cartilage matrix protein (CMP) is a noncollagenous glycoprotein with a molecular mass of 148 kDa consisting of three identical subunits linked together at their C-terminal assembly domains via a coiled-coil α-helix (Hauser and Paulsson 1994). Electron microscopy of the native protein showed trimers with compact ellipsoid subunits, each consisting of two von Willebrand factor A-domains linked via one EGF-like repeat. CMP is a highly insoluble protein which appears to become covalently crosslinked within the cartilage matrix with maturation.

Cartilage oligomeric matrix protein (COMP) has been purified in a native form from articular cartilage (DiCesare et al. 1994a, 1995) and is a pentameric protein consisting of identical subunits with a molecular mass of 110 kDa. COMP belongs to the thrombospondin family and consists of a C-terminal globular domain, followed by seven type III repeats, which have been implicated in calcium binding, four EGF-like repeats and finally an N-terminal assembly domain forming a pentameric coiled-coil α-helix under native conditions. COMP is ubiquitously distributed throughout all different kinds of cartilage and is also found in non-cartilagenous tissues as e.g. tendon (DiCesare et al. 1994b). COMP has been shown to bind chondrocytes (DiCesare et al. 1994a) and could play a role in contacts between extracellular matrix and chondrocytes.

Both COMP and CMP have been used as markers for cartilage degradation in human samples (Lohmander 1994), but studies of their tissue distribution have mainly been done in steer. We therefore undertook to determine their distribution and concentration in human cartilages.

Material and methods

Tissues were obtained in the Department of Forensic Medicine, University of Bern, from recently deceased (within 20 hours) accident or murder victims and used directly.

Extraction of tissues was performed with 4 M guanidine HCl (10 ml per gram of wet tissue) for 16 hours at 4°C after brief homogenization with a Polytron homogenizer.

Polyclonal antisera against bovine CMP and against human COMP, were raised in rabbits.

For immunohistochemistry, sections (5 μm) were cut, digested for 1 h with 40 mU/ml chondroitinase ABC in TBS containing 0.01% (wt./vol.) bovine serum albumin (BSA), incubated in methanol containing 1% (vol./vol.) H_2O_2 and in 1% (wt./vol.) BSA in TBS. Sections were treated with the specific antiserum or nonimmune rabbit serum for 1 hour, followed by peroxidase-conjugated swine anti-rabbit IgG for 45 min. The slides were developed with 0.025% (wt./vol.) 3-amino-9-ethylcarbazole/0.02% (vol./vol.) H_2O_2.

Inhibition ELISA was performed in microtiter plates coated overnight at 4 °C with antigens at 5 mg/ml in TBS and blocked with 1% BSA in TBS for 2 hours at 22–24 °C. Serial dilutions of the extracts were preincubated with the corresponding antibodies, added to the coated plates, and binding of antibodies detected using a secondary antibody to rabbit IgG conjugated with peroxidase and 5-amino-2-hydroxy benzoic acid as a substrate.